Cell PRESS
Immunity
Article

Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms

Baidong Hou,[1] Boris Reizis,[2] and Anthony L. DeFranco[1,*]
[1]Department of Microbiology & Immunology, University of California, San Francisco, CA, 94143, USA
[2]Department of Microbiology, Columbia University Medical Center, New York, NY, 10032, USA
*Correspondence: anthony.defranco@ucsf.edu
DOI 10.1016/j.immuni.2008.05.016

SUMMARY

Toll-like receptors (TLRs) play prominent roles in initiating immune responses to infection, but their roles in particular cell types in vivo are not established. Here we report the generation of mice selectively lacking the crucial TLR-signaling adaptor MyD88 in dendritic cells (DCs). In these mice, the early production of inflammatory cytokines, especially IL-12, was substantially reduced after TLR stimulation. Whereas the innate interferon-γ response of natural killer cells and of natural killer T cells and the Th1 polarization of antigen-specific CD4$^+$ T cells were severely compromised after treatment with a soluble TLR9 ligand, they were largely intact after administration of an aggregated TLR9 ligand. These results demonstrate that the physical form of a TLR ligand affects which cells can respond to it and that DCs and other innate immune cells can respond via TLRs and collaborate in promoting Th1 adaptive immune responses to an aggregated stimulus.

INTRODUCTION

The immune systems of multicellular organisms must recognize the presence of infectious agents and direct effector mechanisms against those agents. In recent years, Toll-like receptors (TLRs), which recognize a variety of pathogen-associated molecular patterns of infectious agents, have emerged as critical for this recognition (Akira et al., 2006). In mammalian tissues, TLRs are highly expressed by resident immune cells, including dendritic cells (DCs), tissue macrophages, and mast cells, and to a lesser degree by other cell types including fibroblasts, epithelial cells, and endothelial cells (Takeda et al., 2003). Upon binding ligands, all known TLRs except for TLR3 can activate downstream signaling cascades through the adaptor protein MyD88 to induce production of inflammatory cytokines by macrophages, DCs, and, to a lesser extent, other cell types. These cytokines, including interferon-α (IFN-α), interferon-β (IFN-β), interleukin 12 (IL-12), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and interleukin 1 (IL-1) attract innate immune cells and/or promote the initiation and polarization of adaptive immune responses (Akira, 2006). Indeed, MyD88-deficient mice have defects in many innate and adaptive immune responses. For example, immunization of mice with TLR ligands, such as CpG or LPS, or with complete Freund's adjuvant, which contains ligands for TLR2 and TLR4, induces naive CD4 T cells to differentiate into T cell helper 1 (Th1) effector cells, and this is severely compromised in mice deficient in MyD88 (Iwasaki and Medzhitov, 2004).

DCs are known to be the main antigen-presenting cells that activate naive T cells in all or most circumstances (Jung et al., 2002; Itano and Jenkins, 2003), but how TLRs promote their maturation and ability to polarize CD4$^+$ T cells to Th1 effector cells is less well understood. During infection, microbial TLR ligands can induce DCs to mature, a response characterized by upregulation of surface major histocompatibility complex (MHC)-peptide complexes and costimulatory molecules involved in activating T cells and of the chemokine receptor CCR7, as well as by migration to the T cell zones in the draining lymph node (Iwasaki and Medzhitov, 2004). Once in lymphoid tissue, mature DCs promote activation of antigen-specific T cells and secrete cytokines and other factors that can promote effector T cell differentiation. However, microbial TLR ligands can also activate other tissue-resident cell types to secrete inflammatory mediators, such as IFN-α, IFN-β, and TNF-α, which can also promote DCs to mature and enable them to promote T cell immune responses, providing an indirect mode of activation of DCs (Kapsenberg, 2003). In order to distinguish the role of direct and indirect modes of TLR activation of DCs, Spörri and Reis e Sousa employed mixed-bone-marrow chimeric mice, which were treated with a synthetic TLR9 ligand. They found that the indirect mode of stimulation was sufficient for maturation of DCs in their experimental system but was insufficient to promote a robust Th1 or Th2 effector cell response (Spörri and Reis e Sousa, 2005). This study suggested that direct TLR stimulation of antigen-presenting DCs, also called "TLR licensing" (Heath and Villadangos, 2005), is important for promoting a robust T cells response even in the context of inflammatory cytokines produced by neighboring cells. To address in more detail the role of TLR signaling in different cell types for immune responses, we created a conditional allele of the mouse *Myd88* gene. By crossing this allele with a DC-specific Cre transgene, CD11cCre (*Itgax*-Cre) (Caton et al., 2007), we have generated highly DC-specific MyD88-deficient mice. Using these mice, we have found that MyD88-dependent signaling in DCs plays a very important role in innate cytokine production and Th1 polarization of antigen-specific CD4$^+$ T cells, but that in some circumstances non-DC cell types can cooperate with DCs to support these immune responses.

Table 1. Deletion Efficiency in DC-*Myd88*$^{-/-}$ Mice

Cell Population	ΔΔCt	Residual *Myd88*^{fl}	% Deletion
myd88$^{fl/fl}$ splenocytes	0	1	0
cDC #1	5.185	0.027	97.3
cDC #2	4.885	0.034	96.6
pDC #1	2.465	0.181	81.9
pDC #2	2.565	0.169	83.1
Mϕ+PMN #1	0.070	0.953	4.7
Mϕ+PMN #2	−0.131	1.095	0*
Mϕ+PMN #3	0.030	0.979	2.1
NK #1	0.110	0.927	7.3
NK #2	−0.115	1.083	0*
NK #3	−0.035	1.025	0*

Quantitative PCR measurement of the amount of the residual "floxed" region in different cell types of several different DC-*Myd88*$^{-/-}$ mice. All cells were FACS purified from spleen. cDC: CD11c^{hi}B220^{-}CD19^{-}. pDCs: CD11c^{int}B220^{+}Ly6C^{+}CD19^{-}. Macrophages and neutrophils (Mϕ+PMN): CD11c^{-}CD11b^{+}. NK cells: NK1.1^{+}TCRβ^{-}. ΔΔCt is the difference of the normalized threshold-cycle number (Ct) between the cell sample of the *Myd88*$^{fl/fl}$CD11c-Cre mice and that of the *Myd88*$^{fl/fl}$ control mice. The values of the residual *Myd88*^{fl} allele were calculated as $2^{-\Delta\Delta Ct}$. % deletion values were calculated as (1 − residual *Myd88*^{fl}) × 100. *, when the calculated number of % deletion was negative, a value of "0" was assigned.

RESULTS

Generation of the *Myd88* Flox Allele and Selective Deletion of MyD88 in DCs

To create a conditional allele of mouse *Myd88*, we introduced 34 base pair LoxP sites on either side of exon 3 of the gene by homologous recombination in embryonic stem cells, which were then used to generate chimeric mice by standard procedures (Figures S1A and S1B available online). Splenic DCs from mice with two copies of this targeted allele expressed MyD88 protein at amounts indistinguishable from wild-type DCs (Figure S1C), indicating that this allele is likely to function normally in the absence of Cre-mediated deletion.

To test the ability of the Cre recombinase to delete exon 3 of the *Myd88* flox *(Myd88*^{fl}*)* allele, we generated mice carrying both the *Myd88*^{fl} allele and the Vav-Cre transgene, which is expressed in all cells of the hematopoietic lineage (de Boer et al., 2003). Splenocytes from *Myd88*$^{fl/fl}$Vav-Cre mice exhibited nearly complete deletion as judged by loss of MyD88 protein expression (Figure S1C).

To study the role of MyD88 in DCs, we crossed mice with the *Myd88*^{fl} allele to mice carrying the CD11c-Cre transgene, which is preferentially expressed in DCs (Caton et al., 2007). The numbers of DCs and of their subsets were unchanged in the spleen and lymph nodes of the *Myd88*$^{fl/fl}$CD11c-Cre mice (Table S2 and data not shown). The deletion of the *Myd88*^{fl} allele was measured by a quantitative PCR assay, which detected the amount of residual *Myd88* exon 3 sequence in genomic DNA. We found that over 94% of the *Myd88*^{fl} allele was deleted in the conventional DCs (cDCs) and around 80% was deleted in plasmacytoid DCs (pDCs), whereas deletion was not detected in splenic macrophages and neutrophils or in naive or activated CD4^{+} T cell (Table 1 and Table S1). These results were corroborated by measurement of MyD88 protein in purified cells (Figure S1D). Natural killer (NK) cells also express CD11c, although at lower amounts than DCs (Laouar et al., 2005). We could not detect deletion in purified NK cells (Table 1). These results demonstrate that there is highly selective deletion in DCs of the *Myd88*$^{fl/fl}$*CD11c-Cre* mice, and, therefore, these mice are referred to here as DC-*Myd88*$^{-/-}$ mice.

Inflammatory-Cytokine Response to TLR Stimulation in the DC-*Myd88*$^{-/-}$ Mice

TLR stimulation induces DCs to produce cytokines, including IL-12, which promotes IFN-γ production and polarization of activated CD4^{+} T cells to the Th1 effector type. To test the importance of MyD88-dependent signaling in DCs for this response, we injected DC-*Myd88*$^{-/-}$ mice and control mice intravenously (i.v.) with CpG-containing oligodeoxynucleotide ODN1826 (CpG), a TLR9 ligand that signals solely through a MyD88-dependent pathway. One hour later, we examined splenic DCs for IL-12 and/or IL-23 production by staining intracellular IL-12p40. The splenic CD8α^{+} DCs in wild-type mice had a robust induction of IL-12p40, but this response was completely defective in the DC-*Myd88*$^{-/-}$ mice (Figure 1A). This result confirmed that ablation of MyD88 function in CD8α^{+} DCs had been achieved and that this rapid response requires MyD88 signaling within DCs. Next, we examined the contribution of DCs to systemic IL-12 production in vivo. Two hours after intravenous injection of CpG, the amounts of IL-12p70 in the serum of DC-*Myd88*$^{-/-}$ mice were below detection limit, whereas the amounts in the control mice increased significantly ($p < 0.01$) (Figure 1B), indicating that the DCs are the major cell type to rapidly produce functional IL-12 in vivo after TLR9 stimulation via the blood stream. Next, we examined the IL-12p40 response of these mice at 1 hr after stimulation with ligands for TLR1+TLR2, TLR4, TLR5, TLR7, or TLR9. We found that the levels of IL-12p40 mRNA induced in total splenocytes in response to stimulation by these TLR ligands administered systemically were all greatly reduced (5–30×) in the DC-*Myd88*$^{-/-}$ mice compared to the control mice (Figure 1C). These results indicate that DCs are the major cell type in the spleen to rapidly produce IL-12p40 in response to the stimulation of most TLRs.

Although DCs are generally thought to be major producers of IL-12, other splenic cell types such as macrophages are also capable of producing substantial amounts of inflammatory cytokines, including IL-1β, IL-6, and TNF-α in response to TLR stimulation. Therefore, we examined the induction of several proinflammatory cytokines after systemic administration of CpG. Surprisingly, we found that DC-*Myd88*$^{-/-}$ mice exhibited substantially reduced mRNA induction of most tested cytokines in the spleen (Figure 1D), as well as greatly decreased amounts of cytokines in the serum (Figure S2 and data not shown). Similar results were also found with ligands for TLR1+TLR2, TLR4, TLR5, and TLR7 (data not shown). These results indicate that DCs play a surprisingly major role in early innate immune cytokine responses upon systemic administration of TLR ligands.

An Aggregated Form of the TLR9 Ligand CpG Induces Distinct Inflammatory Responses

In the experiments described above, we used a synthetic TLR9 ligand that acts in monomeric form (Klinman, 2004). However,

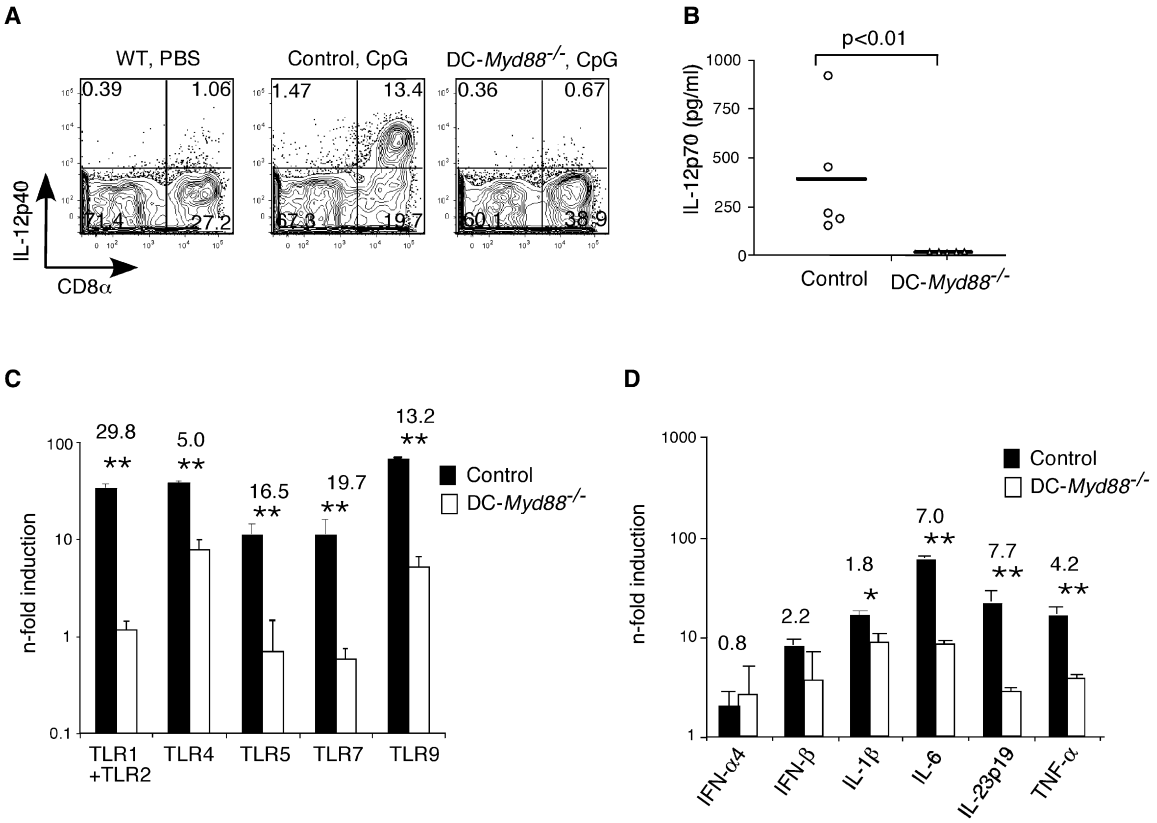

Figure 1. Defective Inflammatory-Cytokine Responses in the DC-*Myd88*$^{-/-}$ Mice

(A and B) IL-12 expression in control or DC-*Myd88*$^{-/-}$ mice after i.v. injection with PBS or CpG. (A) Splenocytes isolated 1 hr after injection were stained for intracellular IL-12p40. Shown are representative FACS plots of gated CD11c^{hi} DCs for one of six mice per group analyzed on two separated days. (B) Amounts of IL-12p70 in the serum of mice, as measured by ELISA, at 2 hr after i.v. injection of CpG (n = 5 for DC-*Myd88*$^{-/-}$ mice). Amounts of IL-12p70 in PBS-treated mice all were under detection limit (not shown).

(C) Induction of IL-12p40 mRNA in the spleen at 1 hr after i.v. injection of Pam3CSK4 (TLR1+TLR2), LPS (TLR4), flagellin (TLR5), CpG (TLR9), or intraperitoneal (i.p.) injection of Imiquimod (TLR7).

(D) Induction of inflammatory-cytokine mRNA in the spleen at 1 hr after i.v. injection of CpG. n-fold induction (mean + standard deviation [SD] of three mice) is relative to the abundance in vehicle-treated mice. Numbers on top of the bars are the n-fold difference between the induction levels in the control and the DC-*Myd88*$^{-/-}$ mice. Statistical comparison is between the DC-*Myd88*$^{-/-}$ mice and the control mice. *, $p < 0.05$; **, $p < 0.01$. Data are representative of two separate experiments.

many natural TLR ligands exist in complexed forms, which could interact differently with host immune cells. To make a complexed form of a TLR ligand, we encapsulated the TLR9 ligand CpG with a cationic lipid, 1,2-dioleoyloxy-3-trimethylammonium-propane-methylsulfate (DOTAP) (hereby referred to as CpG-DOTAP). It has been shown that responses to CpG-DOTAP, like responses to uncomplexed CpG, are dependent on TLR9 and MyD88 (Yasuda et al., 2005)(Honda et al., 2005), a point that we confirmed with *Myd88*$^{-/-}$ mice (data not shown). Using a fluorescently labeled CpG, we found that DOTAP enhanced uptake of CpG in splenic cell types such as CD11b^{+} DCs, pDCs, macrophages, and monocytes (Figure 2A). Complexing CpG with DOTAP also changed the profile of the responding cell types. For example, injecting CpG-DOTAP intravenously induced both splenic CD8α^{+} DCs and CD11b^{+} DCs to express IL-12p40 intracellularly in wild-type mice (Figure 2B), a feature that is similar to LPS, a lipid ligand for TLR4 that also forms aggregated membrane-like structures (Skelly et al., 1979), but unlike soluble CpG (Figure 2B). As expected, the induction of intracellular IL-12p40 was still dependent on DC-intrinsic MyD88 signaling because it was abolished in both CD8α^{+} DCs and CD11b^{+} DCs of the DC-*Myd88*$^{-/-}$ mice (Figure S3A). Interestingly, we also found that CpG-DOTAP induced intracellular TNF-α in CD11b^{+} DCs and in an additional population of cells that were CD11b^{+}F4/80^{+}Ly6C^{+}SSC (side light scatter)^{lo}CD11c^{-}NK1.1^{-}B220^{-}, consistent with a monocyte phenotype (Figure 2C), suggesting that DOTAP also enhances the responses to CpG of other cell types in addition to CD11b^{+} DCs. This TNF-α response was not impaired in the monocytes, but was ablated in DCs in the DC-*Myd88*$^{-/-}$ mice (Figure 2C and Figure S3B), confirming the DC specificity of MyD88 ablation by CD11c-Cre.

Next, to examine a broader range of inflammatory-cytokine responses to this complexed form of TLR9 ligand, we measured inflammatory-cytokine mRNA induction in the spleen after injecting the mice i.v. with CpG-DOTAP. Interestingly, the inductions of many cytokines, including IL-23p19, IL-6, and TNF-α, were attenuated to a much lesser degree in the DC-*Myd88*$^{-/-}$ mice stimulated in this way (Figure 2D) than in mice stimulated with

CpG alone (Figure 1D), suggesting that CpG-DOTAP stimulated TLR9 on non-DC cell types in the spleen more effectively than uncomplexed CpG. In agreement with a previous report (Honda et al., 2005), the CpG-DOTAP complex strongly induced IFN-β and IFN-α4 mRNA (Figure 2D), whereas soluble CpG did so poorly (Figure 1D). By measuring the mRNA induction in different cell types, we found that pDCs were the major type I IFN-producing cell type in the spleen in response to CpG-DOTAP, but other cell types including DCs and macrophages could also produce these cytokines (Table S2). This type I IFN response was abolished in DCs and was substantially reduced in the pDCs in the DC-*Myd88*$^{-/-}$ mice (Table S2), but the overall induction in the spleen was still substantially higher than in the wild-type mice stimulated with CpG alone (Figure 2D).

Role of MyD88 in DCs for the IFN-γ Response to TLR Ligands by NK Cells and NKT Cells

In the early inflammatory-cytokine response to systemic administration of TLR agonists, we consistently observed strongly attenuated IL-12p40 expression in the DC-*Myd88*$^{-/-}$ mice. Because IL-12 is a potent inducer of IFN-γ production, we next asked how IFN-γ production in response to TLR stimulation was affected by ablation of MyD88 selectively in DCs. To identify the cellular source of IFN-γ after TLR stimulation, we stained splenocytes for intracellular IFN-γ at different time points after injecting mice with CpG. Consistent with a previous report (Laouar et al., 2005), we found that NK cells were the major IFN-γ-producing cells in the spleen (Figure S4), starting only a few hours after TLR ligand administration. NKT cells also expressed IFN-γ, but to a substantially lesser degree both in terms of the number of IFN-γ-positive cells in the spleen and of the amount of IFN-γ detected per cell (Figure S4). In contrast, CD4$^+$ T cells, CD8$^+$ T cells, and DCs did not express a large amount of IFN-γ in response to CpG at these early time points. To examine the role of MyD88 signaling in DCs for this in vivo innate IFN-γ response, we measured IFN-γ production by NK cells and NKT cells after TLR stimulation in the DC-*Myd88*$^{-/-}$ mice. Interestingly, we found that the IFN-γ response was totally dependent on MyD88 function in DCs when the mice were stimulated with CpG (Figure 3A and data not shown for NKT cells). Similar results were seen with Pam3CSK4 (TLR1+TLR2 agonist) and Imiquimod (TLR7 agonist) (data not shown). As expected, this IFN-γ response was abolished in mice deficient in IL-12p35 (Figure 3B). These results suggest that the response of NK cells or NKT cells to direct TLR stimulation was not sufficient to produce IFN-γ, and instead these cells needed to be stimulated by cytokines such as IL-12 produced by DCs. When the response to i.v. injection of CpG-DOTAP was examined, the number of IFN-γ-positive cells was substantially enhanced compared to the response to CpG alone (Figure 3A and data not shown for NKT cells). This enhanced IFN-γ response was fully MyD88 dependent and was to a large part dependent on type I IFN signaling (Figure 3B). Interestingly, ablating MyD88 in DCs only partially reduced the IFN-γ response to CpG-DOTAP administered i.v. (Figure 3A), indicating that MyD88 signaling in cell types other than DCs plays a substantial role in this response. Stimulation with LPS also induced a strong IFN-γ response from NK cells in the control mice (Figure 3A, data not shown for NKT cells). This IFN-γ response was also only partially reduced in the DC-*Myd88*$^{-/-}$ mice (Figure 3A), but was almost completely abolished in the *Myd88*$^{-/-}$ mice and in the *Myd88*$^{fl/fl}$*Vav-Cre* mice (Figure S5).

DC Maturation in Response to TLR Stimulation in the DC-*Myd88*$^{-/-}$ Mice

We next assessed the importance of MyD88 signaling in DCs for their ability to prime naive CD4$^+$ T cells. DC maturation in the spleen was examined after the mice were intravenously injected with CpG or CpG-DOTAP. In control mice, both CpG and CpG-DOTAP induced increased expression of CD86, CD40, and, to a lesser extent, class II MHC molecules in splenic cDCs, but the response to CpG-DOTAP was much stronger (Figure 4A). These responses were attenuated in the DC-*Myd88*$^{-/-}$ mice, but in the mice stimulated with CpG-DOTAP, a subset of DCs in the spleen, including both CD8α$^+$ and CD11b$^+$ DCs (data not shown), induced expression of CD86 to a similar degree to that of the DCs from the stimulated MyD88-expressing mice (Figure 4B). These results indicate that cytokines from other cell types surrounding DCs can partially compensate for the loss of direct TLR stimulation to induce some DCs to mature in response to this potent TLR stimulus. It should be noted that the response to CpG-DOTAP remained fully MyD88 dependent because there was no DC maturation in *Myd88*$^{-/-}$ mice (Figure 4B).

Development of CD4$^+$ T Cell Effector Function in the DC-*Myd88*$^{-/-}$ Mice

To examine the effect of defective TLR signaling in DCs on the activation of adaptive immune responses, we adoptively transferred CFSE-labeled ovalbumin (OVA)-specific T cell receptor (TCR)-transgenic OT-II T cells into DC-*Myd88*$^{-/-}$ and control mice and then immunized the mice with OVA by using either CpG or CpG-DOTAP as adjuvants. The proliferation and effector polarization of OT-II T cells in the spleen were analyzed 4 and 7 days later. We found that both immunization protocols led to accumulation of increased numbers of OT-II T cells in the spleen of wild-type recipient mice compared with mice immunized with OVA alone (data not shown). Most of the transferred OT-II T cells had divided multiple times by day 4 after immunization with OVA plus either CpG or CpG-DOTAP, as indicated by CFSE dilution (Figure 5A). However, we consistently found that more OT-II T cells had accumulated in the spleen of the control mice immunized with CpG-DOTAP than in the control mice immunized with CpG (Figure 5B), correlating with the fact that more DCs had matured in the CpG-DOTAP-treated mice (Figure 4). This expansion and survival of antigen-specific naive CD4$^+$ T cells was clearly decreased in the DC-*Myd88*$^{-/-}$ mice immunized with OVA and CpG compared to the control mice (Figure 5B). Similar results were seen on day 7 (data not shown). In contrast, the response was not compromised by MyD88 deficiency in DCs in mice immunized with OVA and CpG-DOTAP (Figure 5B).

In addition, OT-II T cell effector function was evaluated 4 and 7 days after immunization by intracellular staining of IFN-γ after in vitro restimulation for 4 hr. We found that selective deletion of MyD88 in DCs impaired the Th1 effector differentiation of OT-II T cells when CpG was used as adjuvant, as indicated by a reduced number of IFN-γ-producing cells on day 4 (Figure 5), and similarly on day 7 (data not shown). In contrast, no such difference was found when mice were immunized with CpG-DOTAP (Figure 5), suggesting that Th1 differentiation was largely intact in this circumstance

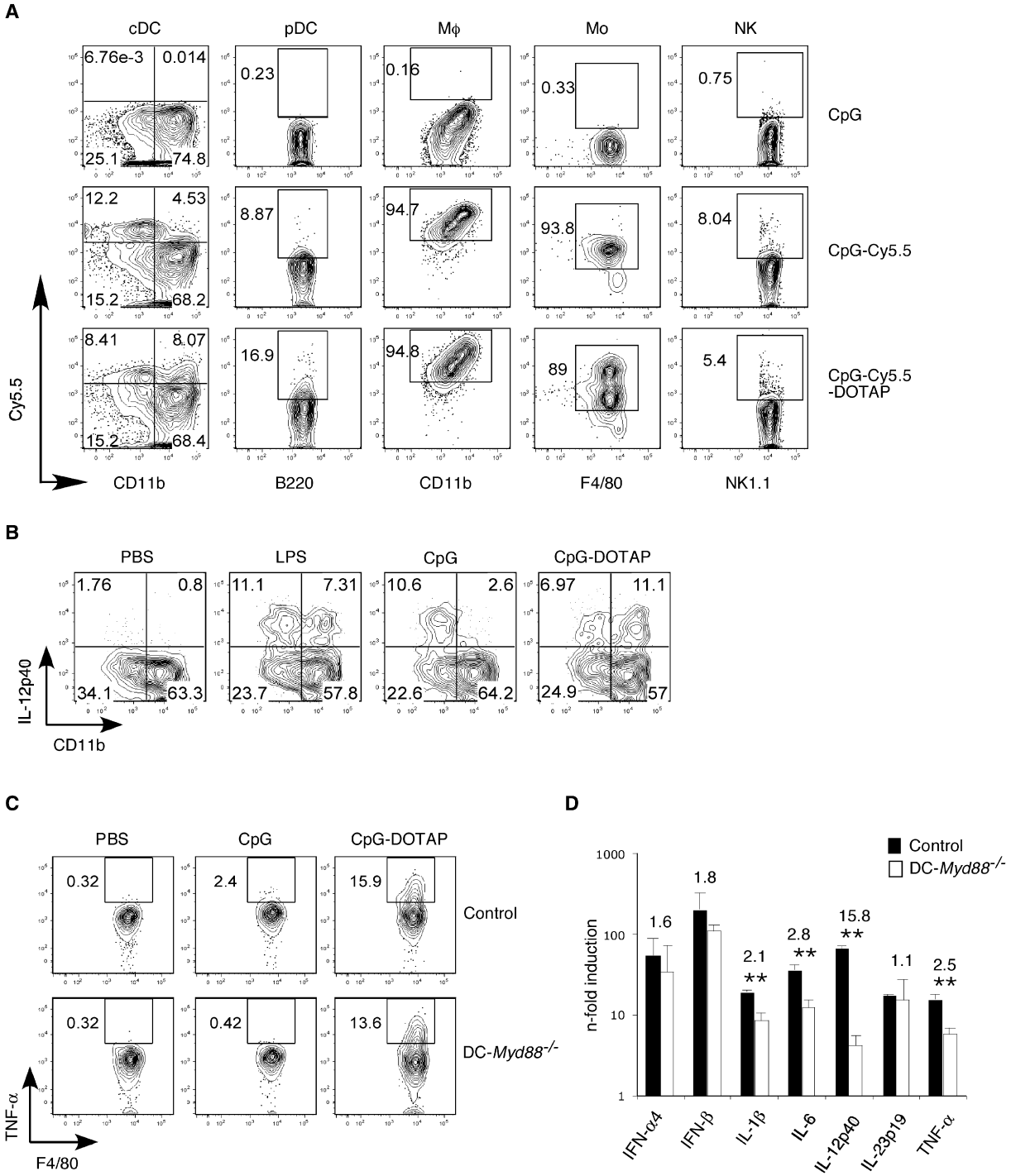

Figure 2. The Physical Form of CpG Influences Its Interaction with Immune Cells

(A) Wild-type mice were injected i.v. with CpG, Cy5.5-labeled CpG, or Cy5.5-labeled CpG complexed with DOTAP. After 30 min, uptake of CpG was examined by flow cytometry on gated conventional DCs (cDCs; CD11c^{hi}I-A^{b+}), plasmacytoid DCs (pDCs; CD11c^{int}B220^{+}Ly6C^{+}CD19^{-}), macrophages (Mϕ; CD11b^{int}F4/80^{hi}Ly6G^{-}SSC^{hi}), monocytes (Mo; CD11b^{+}F4/80^{+}Ly6C^{+}CD11c^{-}Ly6G^{-}SSC^{lo}) or NK cells (NK1.1^{+}TCRβ^{-}) in the spleen. Shown are representative FACS plots for one of two mice per group.

(B) Splenic CD11c^{hi} DCs of wild-type mice were stained for intracellular IL-12p40 1 hr after injection with the indicated TLR ligands. Shown are representative FACS plots for one of four mice per group analyzed on two separate days.

(C) Control or DC-*Myd88*$^{-/-}$ mice were injected i.v. with indicated TLR ligands. After 1 hr, splenocytes were stained for intracellular TNF-α. Shown are representative contour plots of gated CD11b^{+}F4/80^{+}Ly6C^{+}SSC^{lo}CD11c^{-}NK1.1^{-}B220^{-} cells from one of four mice per group analyzed on two separate days.

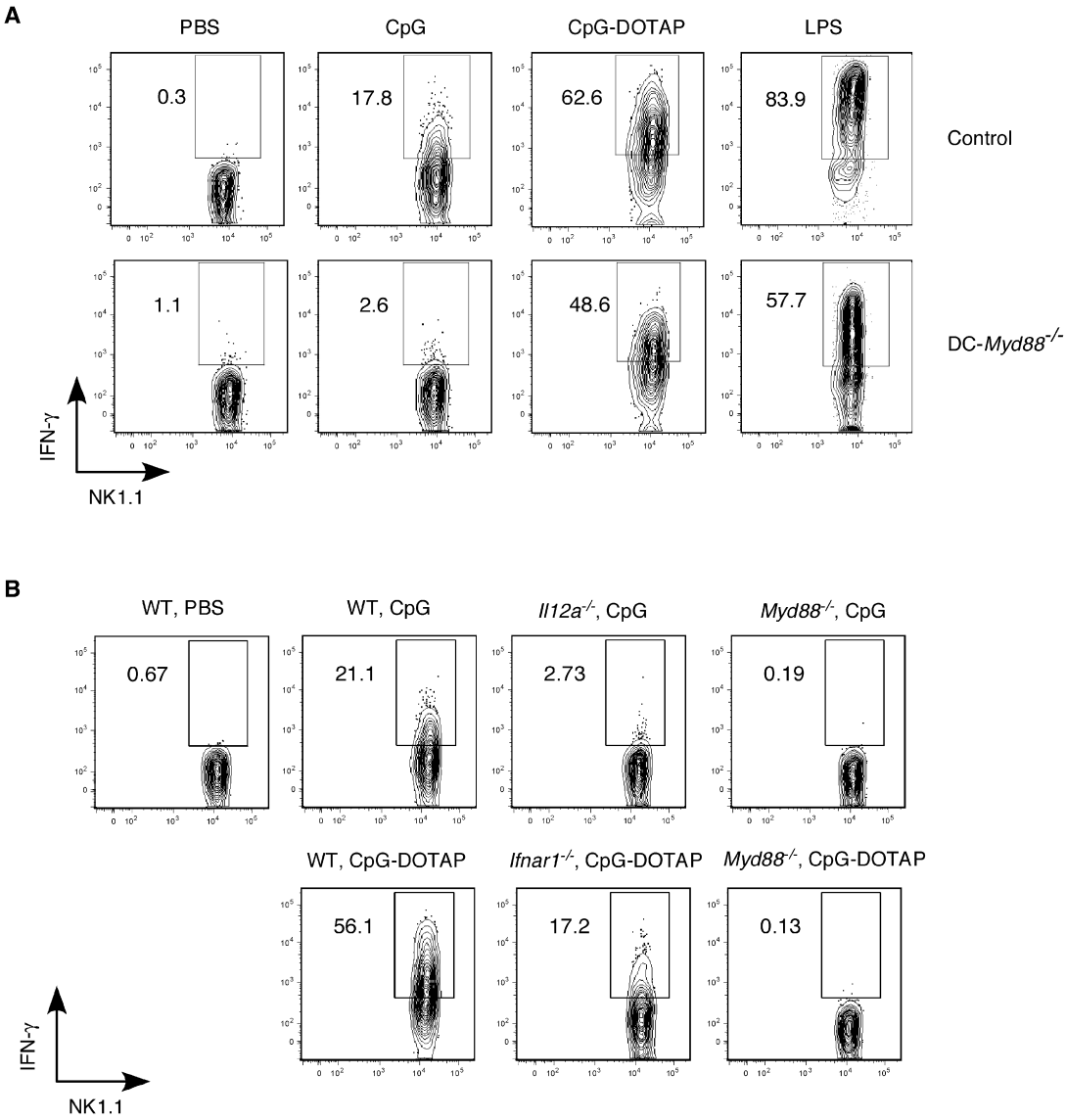

Figure 3. Role of MyD88 in DCs for the IFN-γ Response of NK Cells to TLR Stimulation In Vivo

(A and B) Mice were injected with the indicated TLR ligands. Five hours later, synthesis of IFN-γ by splenic NK cells ($NK1.1^+TCR\beta^-$) was assessed by intracellular staining and flow cytometry. (A) Representative contour plots of control or DC-*Myd88*$^{-/-}$ mice from one of six mice per group analyzed on two separate days. (B) Representative contour plot of wild-type (C57BL/6), *Il12a*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Myd88*$^{-/-}$ mice from one of four mice per group analyzed on two separate days.

where MyD88 signaling in cells other than DCs plays a more prominent role in cytokine production and in induction of DC maturation. Interestingly, mice immunized with OVA and LPS also exhibited strong expansion of OT-II $CD4^+$ T cells and differentiation to Th1 cells in both control and DC-*Myd88*$^{-/-}$ mice (data not shown).

B Cell Antibody Production and Isotype Switching in the DC-*Myd88*$^{-/-}$ Mice

Th1 cells secrete cytokines, including IFN-γ, to induce class switch to IgG2c and IgG2b by responding B cells. As an alternative readout for Th1 development, we examined antibody responses after immunizing mice with OVA plus either CpG or CpG-DOTAP. Although roughly similar amounts of anti-OVA IgM were induced in control and DC-*Myd88*$^{-/-}$ mice, total IgG titers were significantly reduced in DC-*Myd88*$^{-/-}$ mice immunized with OVA and CpG (Figure 6, upper panel). In addition, there was even more drastically reduced production of IgG2c and IgG2b OVA-specific antibody in the DC-*Myd88*$^{-/-}$ mice. In contrast, immunization with OVA and CpG-DOTAP induced comparable amounts of total IgG and IgG1, as well as substantial

(D) Induction of inflammatory-cytokine mRNA in the spleen at 1 hr after injection i.v. with CpG complexed with DOTAP. n-fold induction and n-fold difference were calculated as in Figure 1. Statistical comparison is between the DC-*Myd88*$^{-/-}$ mice and the control mice. *, $p < 0.05$; **, $p < 0.01$. Data are representative of two separate experiments.

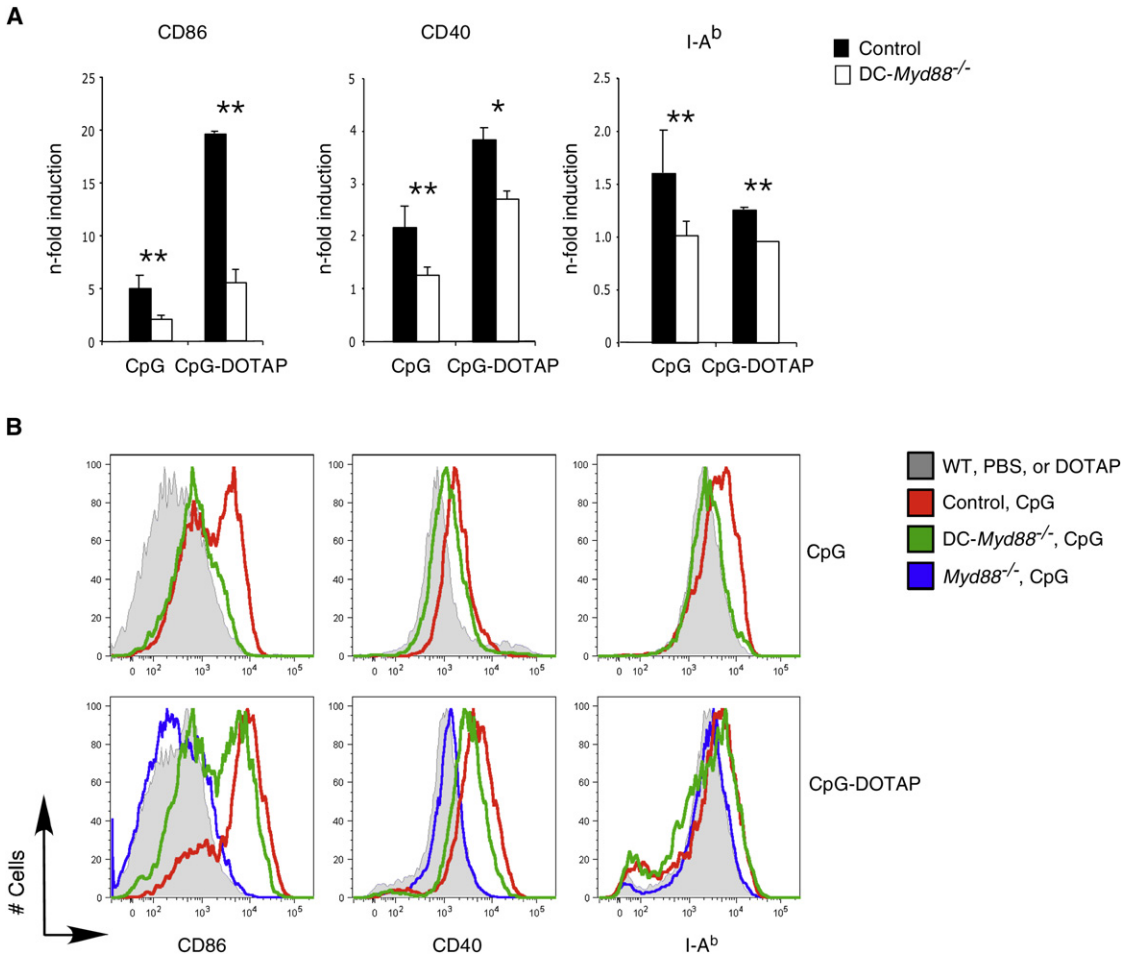

Figure 4. Defects in DC Maturation in the DC-*Myd88*$^{-/-}$ Mice

(A) Control or DC-*Myd88*$^{-/-}$ mice were injected i.v. with CpG or CpG-DOTAP. After 12 hr, expression of CD86 (left), CD40 (middle), or I-A^b (right) on the surface of CD11c^{hi} DCs were assessed by flow cytometry. n-fold induction (mean + SD of four mice) of median fluorescence intensity (MFI) is relative to the expression levels in vehicle-treated mice. Statistical comparison is between the DC-*Myd88*$^{-/-}$ mice and the control mice. *, $p < 0.05$; **, $p < 0.01$.

(B) Representative histograms of CD86 (left), CD40 (middle), or I-A^b (right) on gated CD11c^{hi} DCs from control, *Myd88*$^{-/-}$, or DC-*Myd88*$^{-/-}$ mice 12 hr after i.v. injection of CpG or CpG-DOTAP. Gray histograms represent wild-type mice injected with PBS (upper) or DOTAP (lower).

amounts of IgG2c and IgG2b OVA-specific antibodies in the DC-*Myd88*$^{-/-}$ mice, although the titers were slightly reduced compared to control mice (Figure 6, lower panel). Similar results to those seen with OVA and CpG-DOTAP were seen when mice were immunized with OVA and LPS (data not shown). Thus, as in the case of effector Th1 differentiation, the T cell-dependent IgG response to OVA and CpG was largely dependent on MyD88 function in DCs. However, changing the physical form of the TLR ligand, in this case CpG, to a more aggregated form enhanced cytokine production from other cell types, which probably contributed to the observed T cell-dependent antibody response.

DISCUSSION

In the experiments described here, we have examined the role of TLRs on different immune cell types for the rapid production of inflammatory cytokines and for the activation of the adaptive immune response. Experiments employing a new conditional allele of the gene encoding the key TLR signaling-adaptor molecule, MyD88, together with DC-specific expression of the Cre recombinase, revealed a critical role for TLR signaling in DCs for many responses to TLR ligands. These experiments also revealed situations where other cell types can contribute importantly to the production of certain inflammatory cytokines and relieve the requirement for DC-intrinsic TLR signaling in order to stimulate a vigorous Th1 response.

These experiments provide evidence for the view that the direct recognition of microbial ligands by TLRs on DCs plays a prominent role for the initiation of the adaptive immune response and for directing polarization to a Th1 response. When mice were immunized with ovalbumin and soluble CpG, Th1 polarization of ovalbumin-specific CD4$^+$ T cells was greatly diminished by deletion of the *Myd88* gene selectively in DCs. This result is consistent with a previous study using wild-type and MyD88-deficient bone-marrow chimeras (Spörri and

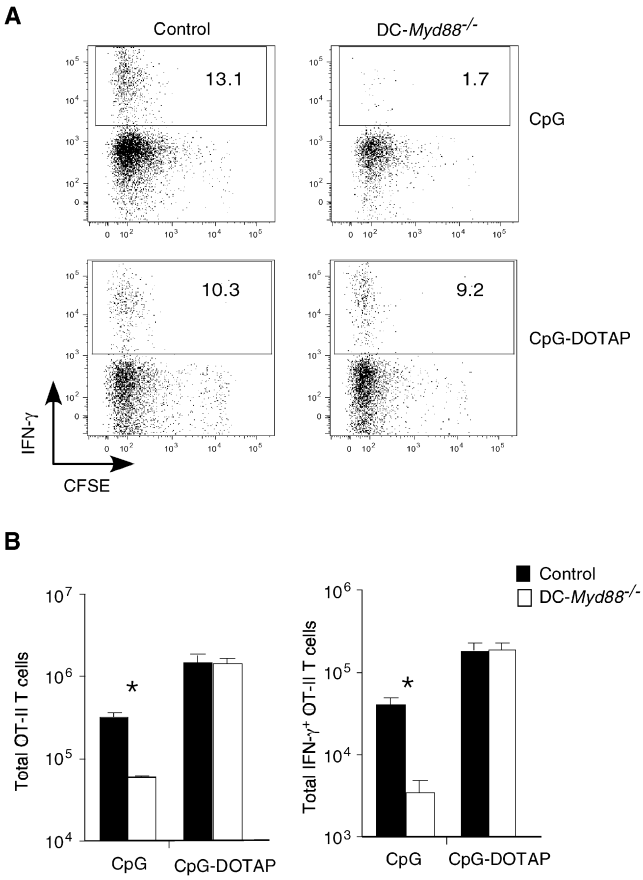

Figure 5. Clonal Expansion and Development of Th1 Effector T Cells in the DC-*Myd88*$^{-/-}$ Mice

(A) Purified CD4$^+$ T cells from OT-II-transgenic mice with a congenic marker (Thy1.1$^+$) were labeled with CFSE and adoptively transferred into Thy1.2$^+$ DC-*Myd88*$^{-/-}$ and control mice. One day later (day 0), the mice were immunized (i.v.) with OVA mixed with CpG (upper panels) or OVA mixed with CpG-DOTAP (lower panels). On days 4 or 7, lymphocytes from the spleen were harvested and restimulated in vitro with PMA and ionomycin for 4 hr. The proliferation of OT-II T cells (identified as CD4$^+$B220$^-$Thy1.1$^+$) was tracked by CFSE dilution, and development of Th1 effector function was assessed by intracellular IFN-γ staining and flow cytometry. Shown are representative dot plots of intracellular IFN-γ staining and CFSE dilution on day 4 of single mice immunized with OVA and CpG or OVA and CpG-DOTAP. Numbers in the gates indicate percentage of OT-II T cells that were IFN-γ$^+$.

(B) Absolute numbers of OT-II T cells (left panel) and IFN-γ$^+$ OT-II T cells (right panel) in the spleen at day 4 after immunization with OVA and CpG or OVA and CpG-DOTAP and in vitro restimulation, as calculated from the counts of total splenocytes and the percentages of OT-II T cells and IFN-γ$^+$ OT-II T cells in the spleens. Data are expressed as mean + SD of four mice and are representative of three separate experiments. Statistical comparison is between the DC-*Myd88*$^{-/-}$ mice and the control mice. *, $p < 0.05$.

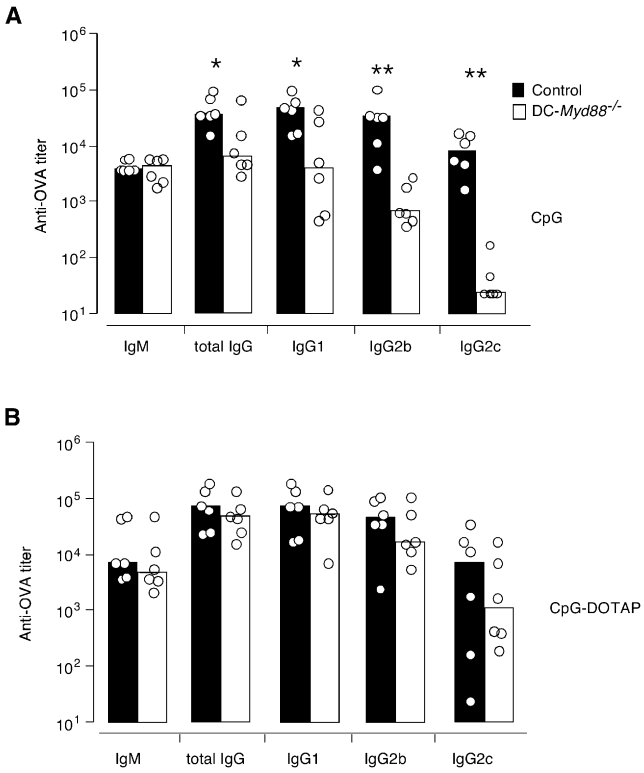

Figure 6. Antibody Response in the DC-*Myd88*$^{-/-}$ Mice

Control and DC-*Myd88*$^{-/-}$ mice were immunized i.p. with OVA together with CpG (A) or CpG-DOTAP (B) and were bled 7 and 14 days after immunization. Sera were serially diluted, and OVA-specific antibody isotypes (IgM on day 7 and total IgG, IgG1, IgG2b, and IgG2c on day 14) were measured by ELISA. Data shown are the end-point titers of all samples. Statistical significance was calculated with Mann-Whitney U test. *, $p < 0.05$ **, $p < 0.01$. Similar results were obtained in three (CpG) and two (CpG-DOTAP) separate experiments.

Reis e Sousa, 2005). However, in contrast to that study, we found that DCs lacking MyD88 had substantially compromised maturation in response to soluble CpG administered i.v., as indicated by the reduced induction of costimulatory molecules. A major difference between those experiments and the ones described here is that in the bone-marrow chimeric mice, there was a mixture of MyD88-expressing DCs and MyD88-deficient DCs, whereas in the experiments described here, the vast majority of the DCs were MyD88 deficient and other cell types retained MyD88 expression. Therefore, the indirect maturation observed in the bone-marrow chimeric mice probably reflects the action of cytokines produced primarily by neighboring DCs, acting in a paracrine manner. This interpretation is supported by observations reported here that DCs were the major cell types producing cytokines in response to soluble TLR ligands. Taken together, our results and those of Spörri and Reis e Sousa (2005) demonstrate the importance of TLR signaling in DCs for CD4$^+$ T cell responses, at least in the context of immunization with soluble TLR ligands.

MyD88 function in DCs was found to be especially important for IL-12 production in response to TLR ligand stimulation. IL-12 is known to stimulate innate immune cell types such as NK cells to express IFN-γ and to promote the Th1 polarization of CD4$^+$ T cells (Magram et al., 1996), both of which can be enhanced by IL-18 (Takeda et al., 1998). Indeed, we found that the early IFN-γ production from NK cells and NKT cells in response to soluble CpG was totally dependent on IL-12. IFN-γ from NK cells can initiate Th1 polarization of antigen-stimulated CD4$^+$ T cells (Martin-Fontecha et al., 2004) by inducing the fate-determining transcription factor T-bet (Afkarian et al., 2002).

In addition, IL-12 from DCs promotes fate stabilization of T cells polarizing to Th1 cells by promoting their secretion of IFN-γ. Given the critical roles of IL-12 and the fact that its induction by TLR ligands was highly dependent on direct TLR stimulation in DCs, it is very likely that IL-12 was an essential cytokine for promoting Th1 polarization under these circumstances. Thus, TLR-MyD88 signaling in DCs probably contributes to adaptive Th1 immune responses to immunization with soluble antigen and TLR ligands in two main ways: by contributing to DC maturation and by inducing IL-12 production.

We found that the physical form of the TLR ligand had a large effect on the ability of different cell types to contribute to the immune response in vivo. CpG presented in an aggregated form by being complexed with the cationic lipid DOTAP induced a potent type I IFN response compared to CpG alone, as reported previously (Honda et al., 2005). In contrast to the strong dependence of MyD88 function in DCs on the cytokine responses to soluble CpG, MyD88 function in both conventional DCs and other cell types made important contributions to this response. Indeed, whereas when soluble CpG was injected i.v., it was primarily $CD8\alpha^+$ DCs in the spleen that made cytokines initially, when CpG-DOTAP was injected i.v., both $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets responded rapidly, as did a $F4/80^+$ cell type that may be the inflammatory monocyte. In addition, pDCs were responsible for most of the type I IFN production in response to CpG-DOTAP. Interestingly, the uptake of CpG in these experiments was substantially enhanced when CpG was complexed with DOTAP. The altered spectrum of responding cell types may relate to changes in the mechanism of cell uptake, and it has been reported that CpG-DOTAP complexes enter cells through the endocytic pathway (Zabner et al., 1995). Interestingly, interaction of self-DNA with a cationic amphipathic antimicrobial peptide LL37 has recently been implicated in the pathogenesis of psoriasis. This complex, which may be similar in its action to the CpG-DOTAP aggregate studied here, was found to greatly enhance activation of pDCs in the affected skin of patients with psoriasis (Lande et al., 2007). It is likely that many microbial and endogenous TLR ligands exist in aggregated or particulate forms, so the ability of both DCs and other myeloid cell types to respond to TLR ligands presented in these complex forms may be relevant to many biological situations.

The ability of CpG-DOTAP to induce large amounts of type I IFNs from pDCs, as well as from other non-DC cell types, is likely to explain its ability to induce strong IFN-γ production from NK cells, robust DC maturation, and a vigorous Th1 response in the absence of MyD88 expression in DCs. Other have reported that type I IFNs can synergize with IL-18 to induce IFN-γ even in IL-12-deficient splenocyte cultures (Freudenberg et al., 2002), and we found that the NK cell IFN-γ response to CpG-DOTAP was largely dependent on the expression of type I IFN receptors. In addition, type I IFNs are known to be able to induce maturation of DCs (Hoebe and Beutler, 2004). Indeed, it has been shown that coadministration of IFN-α with antigen induces delayed-type hypersensitivity (Gallucci et al., 1999), as well as IgG2a antibody production (Le Bon et al., 2001), both of which are typical Th1 responses. Thus, the robust production of type I IFNs by cell types other than conventional DCs is likely to explain why MyD88 function in DCs was not necessary for DC maturation or for a vigorous Th1 response in DC-$Myd88^{-/-}$ mice immunized with OVA and CpG-DOTAP.

In our experiments, LPS had a behavior that was very similar to that of CpG-DOTAP. LPS is an amphipathic component of gram-negative bacterial cell walls that forms large aggregates in solution, so it is not surprising that it has the ability to induce robust cytokine responses from DC subsets and also from additional myeloid cell types similarly to CpG-DOTAP. However, it is worth noting that LPS, like CpG-DOTAP, neither induced IFN-γ from NK cells and NKT cells (Figure S5) nor promoted Th1 polarization in $Myd88^{-/-}$ mice (Schnare et al., 2001). Although TLR9 and TLR4 both signal via MyD88, TLR4 also signals via the TRIF adaptor, and this pathway induces abundant type I IFNs. Indeed, previous work showed that LPS stimulation of $Myd88^{-/-}$ mice failed to promote Th1 responses despite a vigorous type I IFN response and clearly evident DC maturation (Pasare and Medzhitov, 2004). Thus, type I IFN production alone is insufficient to drive Th1 responses. These results suggest that one or more MyD88-dependent cytokines in addition to type I IFNs coming from cells other than DCs are also required for the innate IFN-γ and Th1 response seen in DC-$Myd88^{-/-}$ mice immunized with CpG-DOTAP or LPS. IL-18 is known to synergize with type I IFNs or IL-12 to induce IFN-γ (Freudenberg et al., 2002; Nakanishi et al., 2001) and therefore is a strong candidate for the additional MyD88-dependent cytokine required for these responses.

In any case, our results clearly show that the functional fate of a T cell is not only affected by the DC that the T cell is interacting with, but also by surrounding cells responding via TLRs and MyD88. These surrounding cells include other DCs and non-DC cell types in the infected tissues. This *trans* effect may allow cooperation between cell types expressing different sets of TLRs. For example, $CD8\alpha^+$ DCs, which do not express TLR7 (Edwards et al., 2003), can receive important cytokine signals from pDCs, which secrete type I IFNs after TLR7 stimulation, allowing them to activate T cells. Other recent studies have also provided evidence for regulatory effects of macrophages on DCs for polarizing T cell differentiation. For example, lamina propria macrophages from the gut were found to express anti-inflammatory cytokines even after TLR stimulation in vitro and to promote development of $FoxP3^+$ regulatory T cells, which restrain immune responses to commensal microbes and dietary antigens, whereas DCs from the same location responded to the same stimulus by producing proinflammatory cytokines and promoting IL-17-producing T cell responses (Denning et al., 2007). Clearly, much remains to be learned about how different types of innate immune cells communicate with one another and combine to direct the nature of the adaptive immune responses.

In summary, our results indicate that MyD88-dependent signaling in both DCs and non-DC cell types can support Th1 differentiation depending on the type of TLR stimulation. Whereas direct TLR stimulation is likely to be the most efficient way for activating DCs and for activating adaptive responses, we have found that other cell types stimulated with TLR ligands in complex forms secrete substantial amounts of cytokines that can make important contributions to both innate and adaptive immune responses. It should be very interesting to use the mice described here to dissect further the role of TLR signaling in different cell types for activation of adaptive immune responses in more complicated and biologically important situations, such as infections with pathogens and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Generation of the *Myd88^{fl}* Allele

A conditional allele of *Myd88* was created in mouse E14 embryonic stem cells (ESCs) following standard procedures (see Supplemental Experimental Procedures for details). Out of 400 ESC clones screened, five had recombined into the endogenous *Myd88* locus. Three independent lines of the homologously recombined ESCs were injected into blastocysts from B6 mice by the UCSF mouse genetic core facility, and animals with high levels of incorporation of the 129 ESCs into the embryo were obtained. The mice were bred, and germline transmission of the targeted allele was obtained in mice originating from the three ESC lines. The offspring were then bred to ACTB-FLPe mice, which express FLP recombinase (Rodriguez et al., 2000), to remove the neomycin resistance cassette. This leaves the conditional allele with two loxP sites and one residual FRT site.

Mice

B6 (000664; C57BL/6J) and B6-Thy1.1 (001317; B6.Cg-Igha Thy1a Gpi1a/J) mice were from Jackson Laboratory. ACTB-FLPe mice (Rodriguez et al., 2000) were obtained from G. Martin (UCSF). CD11c-Cre mice have been described (Caton et al., 2007), and the mice used in this study were backcrossed to B6 for at least six generations. Vav-Cre mice (de Boer et al., 2003) were a gift of D. Kioussis (National Institute for Medical Research, London, UK). *Myd88$^{-/-}$* mice (Adachi et al., 1998) were originally from S. Akira (Osaka University, Osaka, Japan) and were backcrossed to B6 for ten generations in our colony. Mice deficient in IL-12p35 (*Il12a$^{-/-}$*) (Mattner et al., 1996) were obtained from R. Locksley (UCSF). *Ifnar1$^{-/-}$* mice (Muller et al., 1994) were obtained from M. Matloubian (UCSF). OT-II mice (Barnden et al., 1998) were bred to B6-Thy1.1 mice, and the F1 male mice were used as the donors of TCR-transgenic CD4 T cells.

The *Myd88^{fl}* mice used in this study were backcrossed to B6 for at least six generations and then crossed to CD11c-Cre mice or Vav-Cre-transgenic mice. In this study, *Myd88$^{fl/fl}$* or *Myd88$^{fl/-}$* mice containing the CD11c-Cre transgene or the Vav-Cre transgene were used, and *Myd88$^{fl/fl}$* or *Myd88$^{fl/-}$* mice were used as controls.

All experimental mice were used at 8–12 weeks of age and were sex matched and age matched (within 2 weeks) within experiments. All animals were housed in a specific pathogen-free animal facility at UCSF under conditions that meet institutional animal care and use committee (IACUC) and National Institutes of Health (NIH) guidelines.

Reagents

CpG oligodeoxynucleotide 1826 containing a phosphorothioate backbone (CpG) and Cy5.5-labeled CpG were purchased from Integrated DNA Technologies (IDT). For making the CpG-DOTAP complex, 25 μg of CpG was diluted in 75 μl of 20 mM HEPES-buffered saline (HBS) and then mixed with 50 μg of DOTAP (Roche) diluted to 75 μl with HBS for 15 min before injection. Ultrapure LPS (*E.coli* 0111:B4), Pam3CSK4, and imiquimod (R837) were purchased from Invivogen. *Salmonella typhimurium* flagellin was purified from a *fljB^{-} fliC^{+}* strain (TH4778, kindly provided by K. Hughes, University of Utah, UT), following a protocol provided by K.D. Smith (University of Washington, WA). Chicken ovalbumin was purchased from Sigma-Aldrich, and endotoxin was removed by Triton X-114 treatment as described (Aida and Pabst, 1990). All reagents were free of endotoxin as determined by the Limulus Amebocyte Lysate Test (BioWhittaker).

Quantitative PCR

For assessing the deletion efficiency in particular cell types of the DC-*Myd88$^{-/-}$* mice, genomic DNA was extracted from FACS-purified cells, and the residual amount of the "floxed" region was quantified by Taqman PCR (see Supplemental Experimental Procedures for details). The amount of flox allele of each mouse was normalized to Edg-1. The genomic DNA from *Myd88$^{fl/fl}$* mice was used for the no-deletion control.

For quantifying cytokine induction after systemic administration of TLR ligands, mouse spleens were harvested and snap frozen in liquid nitrogen. Total RNA was extracted with the RNeasy kit (QIAGEN) with on-column DNase digestion. cDNA was transcribed from total RNA with the iScript cDNA Synthesis Kit (Bio-Rad). Transcripts were quantified by PCR with iTaq SYBR Green Supermix with ROX (Bio-Rad), and the levels of cytokine transcripts were normalized to the levels of HPRT mRNA. The induction of cytokine mRNA was expressed as a ratio between the mRNA value of the TLR-ligand-treated mice and that of the vehicle-treated control mice. All primers (Table S3) were obtained from IDT.

Enzyme-Linked Immunosorbent Assay

The amounts of cytokines in serum were analyzed by standard sandwich enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences). The titers of OVA-specific immunoglobulin isotypes in serum were determined by ELISA with horseradish peroxidase (HRP)-conjugated anti-mouse IgM, total IgG, IgG1, IgG2b, or IgG2c reagents (Southern Biotech) to detect immunoglobulin bound to the OVA-coated plates. Antibody titers were determined as the reciprocal of the dilution that gave an optical density value (450–570 nm wavelength) that was more than ten times the standard deviation above the mean value of the negative-control wells.

Flow Cytometry

For surface staining of DCs, single-cell suspensions were prepared from spleens with the digestion medium (see Supplemental Experimental Procedures). The cells were then stained in ice-cold FACS buffer (PBS supplemented with 2 μM EDTA, 1% heat-inactivated FCS, and 0.02% sodium azide). Anti-CD16/CD32 (Ab) (2.4G2, BD PharMingen) was used to block nonspecific antibody binding. All fluorochrome-conjugated monoclonal antibodies were purchased from BD PharMingen or eBioscience.

To detect in vivo IL-12, TNF-α, or IFN-γ expression in particular cell types, splenocytes were prepared, and surface markers were stained as described above with the exception that all media contained 10 μg/ml brefeldin A (Sigma-Aldrich). Then the cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences). Intracellular IL-12 was detected by phycoerythrin- or allophycocyanin-labeled IL-12p40/p70-specific antibody (C15.6, BD PharMingen), TNF-α was detected by phycoerythrin-labeled TNF-α-specific antibody (MP6-XT22, BD PharMingen), and IFN-γ was detected by allophycocyanin-labeled IFN-γ-specific antibody (XMG1.2 BD PharMingen).

For assessment of ex vivo T cell IFN-γ expression, single-cell suspensions were prepared from the spleen, and 5×10^6 cells/ml were cultured for 4 hr in complete RPMI-1640 medium (10% heat-inactivated FCS, 25 mM HEPES, 1 mM L-glutamine, 50 μM 2-mercaptoethanol) containing 100 pg/ml PMA and 1 ng/ml ionomycin. Brefeldin A (10 μg/ml) was added for the last 2 hr of culture. Then the intracellular IFN-γ was stained as described above.

All data were collected on a LSRII flow cytometer (Becton Dickinson) and were analyzed with FlowJo software (TreeStar).

Adoptive Transfer

Purified OT-II T cells (5×10^5) were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) for 8 min at 37°C and then transferred into sex-matched recipient mice by intravenous injection 1 day before immunization.

In Vivo Stimulation of TLRs and Immunization

For in vivo stimulation, mice were injected with TLR ligands either intravenously (CpG 25 μg, Cy5.5-labeled CpG 25 μg, LPS 25 μg, Pam3CSK4 50 μg, flagellin 20 μg, or CpG-DOTAP complex 25 μg/50 μg) or intraperitoneally (imiquimod 150 μg).

Mice were immunized with 50 μg OVA mixed with 25 μg CpG or with CpG-DOTAP (25 μg/50 μg) intraperitoneally for antibody response, or intravenously for T cell responses. Sera and lymphoid organs were collected at the indicated times.

Statistical Analysis

Statistical significance was calculated with an unpaired Student's t test or Mann-Whitney U test. All p values of 0.05 or less were considered significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, three tables, and five figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/2/272/DC1/.

ACKNOWLEDGMENTS

We thank N. Killeen, V. Nguyen, and A. Kuroda for assistance in creating the *Myd88*^{fl} allele; W. Hazenbos for providing the Vav-Cre mice; S. Watson, J. Jarjoura, and C. McArthur for assistance with cell purification by flow cytometry; L. Kuzmich and J. Lyandres for assistance with the mouse colonies; Y. Xu for helping with RT-PCR; L. Lee for providing low LPS flagellin; A. Abbas, J. Bluestone, Z. Hua, L. Lanier, and R. Locksley for helpful discussions; A. Gross for comments; and C. Lowell and L. Fong for critical reading of the manuscript. This research was supported by awards from the Academic Senate of UCSF and from the Sandler Foundation to A.L.D., and by the NIH (R01 AI072058). B.H. is a recipient of an Arthritis Foundation Postdoctoral Fellowship. B.R. is supported by an award from the Sandler Foundation and by the NIH (AI067804).

Received: February 6, 2008
Revised: May 13, 2008
Accepted: May 23, 2008
Published online: July 24, 2008

REFERENCES

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity *9*, 143–150.

Afkarian, M., Sedy, J.R., Yang, J., Jacobson, N.G., Cereb, N., Yang, S.Y., Murphy, T.L., and Murphy, K.M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. Nat. Immunol. *3*, 549–557.

Aida, Y., and Pabst, M.J. (1990). Removal of endotoxin from protein solutions by phase separation using Triton X-114. J. Immunol. Methods *132*, 191–195.

Akira, S. (2006). TLR signaling. Curr. Top. Microbiol. Immunol. *311*, 1–16.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783–801.

Barnden, M.J., Allison, J., Heath, W.R., and Carbone, F.R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol. Cell Biol. *76*, 34–40.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J. Exp. Med. *204*, 1653–1664.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J., and Kioussis, D. (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur. J. Immunol. *33*, 314–325.

Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R., and Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat. Immunol. *8*, 1086–1094.

Edwards, A.D., Diebold, S.S., Slack, E.M., Tomizawa, H., Hemmi, H., Kaisho, T., Akira, S., and Reis e Sousa, C. (2003). Toll-like receptor expression in murine DC subsets: Lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur. J. Immunol. *33*, 827–833.

Freudenberg, M.A., Merlin, T., Kalis, C., Chvatchko, Y., Stubig, H., and Galanos, C. (2002). Cutting edge: A murine, IL-12-independent pathway of IFN-gamma induction by gram-negative bacteria based on STAT4 activation by Type I IFN and IL-18 signaling. J. Immunol. *169*, 1665–1668.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: Endogenous activators of dendritic cells. Nat. Med. *5*, 1249–1255.

Heath, W.R., and Villadangos, J.A. (2005). No driving without a license. Nat. Immunol. *6*, 125–126.

Hoebe, K., and Beutler, B. (2004). LPS, dsRNA and the interferon bridge to adaptive immune responses: Trif, Tram, and other TIR adaptor proteins. J. Endotoxin Res. *10*, 130–136.

Honda, K., Ohba, Y., Yanai, H., Negishi, H., Mizutani, T., Takaoka, A., Taya, C., and Taniguchi, T. (2005). Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature *434*, 1035–1040.

Itano, A.A., and Jenkins, M.K. (2003). Antigen presentation to naive CD4 T cells in the lymph node. Nat. Immunol. *4*, 733–739.

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nat. Immunol. *5*, 987–995.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., et al. (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211–220.

Kapsenberg, M.L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. Nat. Rev. Immunol. *3*, 984–993.

Klinman, D.M. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. *4*, 249–258.

Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y.H., Homey, B., Cao, W., Wang, Y.H., Su, B., Nestle, F.O., et al. (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature *449*, 564–569.

Laouar, Y., Sutterwala, F.S., Gorelik, L., and Flavell, R.A. (2005). Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. Nat. Immunol. *6*, 600–607.

Le Bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F., and Tough, D.F. (2001). Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity *14*, 461–470.

Magram, J., Connaughton, S.E., Warrier, R.R., Carvajal, D.M., Wu, C.Y., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D.A., and Gately, M.K. (1996). IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity *4*, 471–481.

Martin-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2004). Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat. Immunol. *5*, 1260–1265.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Di Padova, K., Behin, R., Gately, M.K., Louis, J.A., and Alber, G. (1996). Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. Eur. J. Immunol. *26*, 1553–1559.

Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. Science *264*, 1918–1921.

Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. (2001). Interleukin-18 regulates both Th1 and Th2 responses. Annu. Rev. Immunol. *19*, 423–474.

Pasare, C., and Medzhitov, R. (2004). Toll-dependent control mechanisms of CD4 T cell activation. Immunity *21*, 733–741.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139–140.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., and Medzhitov, R. (2001). Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. *2*, 947–950.

Skelly, R.R., Munkenbeck, P., and Morrison, D.C. (1979). Stimulation of T-independent antibody responses by hapten-lipopolysaccharides without repeating polymeric structure. Infect. Immun. *23*, 287–293.

Spörri, R., and Reis e Sousa, C. (2005). Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nat. Immunol. *6*, 163–170.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu. Rev. Immunol. *21*, 335–376.

Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. (1998). Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity *8*, 383–390.

Yasuda, K., Yu, P., Kirschning, C.J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., and Wagner, H. (2005). Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J. Immunol. *174*, 6129–6136.

Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., and Welsh, M.J. (1995). Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. *270*, 18997–19007.